

Purification and Characterization of a Human Membrane Protein That Activates the Alternative Complement Pathway and Allows the Deposition of Homologous Complement C3

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Summary

A human myeloid cell subline, P39⁺, is found to be a target for human complement (C) via the alternative pathway and to allow the deposition of multiple C3 fragments on its membranes, though expressing the complement regulatory proteins decay-accelerating factor and membrane cofactor protein. The parent cell line, P39⁻, which is phenotypically similar to the P39⁺ subline, does not allow the deposition of homologous C3 fragments. In this study, we established a monoclonal antibody, M161 Ab, which reacted with P39⁺ but not P39⁻ cells. This Ab recognized a 43-kD protein in P39⁺ cell lysate transblotted onto nitrocellulose. Using this Ab as a probe, we purified the 43-kD protein, namely, M161 antigen (Ag). M161 Ag had a basic isoelectric point (pI), 9.3–9.4 by chromatofocusing, and was precipitated as an insoluble material at the pI point. The purified M161 Ag was a single-chain protein and did not possess N- or O-linked carbohydrates. When the purified M161 Ag was transblotted onto nitrocellulose and incubated with Mg²⁺-EGTA serum, human C3 fragments were efficiently deposited on M161 Ag. The major species of the deposited C3 fragments was C3b. Furthermore, the C3 fragments bound to the M161 Ag were detached by 1 M hydroxylamine, suggesting that a covalent ester linkage sustains M161 Ag–C3b interaction. NH₂-terminal amino acid analysis revealed that M161Ag is a novel membrane protein. Hence, it appeared that M161 Ag is a potent activator of human alternative complement pathway on human cells that activates homologous C3 and allows the deposition of C3b on itself. Thus, under some conditions, homeostasis of complement is maintained even on human cells, not only by the complement regulatory proteins, but also by membrane C3-activating molecules on which C3b is deposited.

The alternative complement pathway has been characterized as a humoral system of natural immunity to invading pathogenic microorganisms and virus-infected cells (1). Recognition and subsequent activation of the alternative C pathway results in the covalent attachment of C3 fragments to target membranes, which facilitates interaction of the pathogens with C3 receptor-bearing host effector cells and successive C activation into target cell lysis (2). C3 deposition is usually observed in foreign material, and the molecules responsible for the acceptance of human C3b have been identified (3–6). As an exceptional event, human C3 is deposited on human B lymphoid cells bearing CR2 (7–9), the mechanism and physiological role of which largely remain to be elucidated.

The mechanism of C3 activation and deposition on biological targets has been thoroughly investigated and addressed as the reaction involving the cleavage of the intramolecular

thioester bond of native C3 accompanied by proteolytic activation of C3 to C3b and formation of a covalent linkage of C3b with hydroxyl or amino groups on target molecules (2). C3 activation is amplified by the deposited C3b via the alternative pathway (2). Regulation of C3 fragment deposition is controlled by serum and membrane-bound complement regulatory proteins, factor H (10), properdin (11), CR1 (12), decay-accelerating factor (DAF)¹ (13), and membrane cofactor protein (MCP) (14), as well as membrane constituents such as sialic acids (15) and gangliosides (16).

We demonstrated that a human myeloid cell subline,

¹ Abbreviations used in this paper: C3_{MA}, methylamine-treated C3; DAF, decay-accelerating factor; GVB, gelatin veronal buffer; HRP, horseradish peroxidase; MCP, membrane cofactor protein; NHS, normal human serum; p-APMSF, (p-amidinophenyl)methanesulfonyl fluoride hydrochloride.

P39⁺, is a target for homologous complement attack via the alternative pathway despite the expression of normal DAF and MCP (17), and that they expressed C3-activating membrane molecules distinct from CR2 (18), which is the only identified molecule capable of inducing C3 deposition on some Burkitt's lymphoma cell lines (8). In addition, P39⁺ cells tend to self-aggregate, whereas parent normal P39 cells (designated as P39⁻ cells) grow separately (18). On P39⁺ cells but not P39⁻ cells, C3b remained undegraded for relatively long periods when associated with these membrane proteins, and significant amounts of C5a are released when P39⁺ cells are incubated with human serum containing Mg²⁺-EGTA (the source of the alternative C pathway) (18). P39⁻ cells do not activate homologous C (18). Thus, in P39⁺ cells, the C3b bound to the membrane proteins must have the ability to assemble in the C3/C5 convertase, thus overcoming the regulatory functions of factor H, DAF, and MCP. In this study, we developed a mAb that reacted with P39⁺ but not P39⁻ cells. Using this mAb as a probe, we purified a P39⁺ cell membrane protein, which is a homologous C3-activating molecule on human cells.

Materials and Methods

Cells, Antibodies, and Reagents. Human myeloid cell lines P39⁻, KG-1, and THP1 were provided by the Japanese Cancer Research Resources Bank, and HL60 and U937 were gifts from Dr. J. P. Atkinson (Washington University, St. Louis, MO). The P39⁺ subline was established as described (18). The cells were usually maintained in RPMI-1640 supplemented with 10% FCS (Cell Culture Laboratories, Cleveland, OH) in the presence of antibiotics. For large scale culture, the P39⁺ cells were cultured in Hymedium 910B supplemented with 2% FCS (Nipro Co., Osaka, Japan). Cultures were kept in an atmosphere of 5% CO₂/95% air at 37°C. Two sublines of P39, P39⁺ and P39⁻, were maintained as previously described (18). P39⁻ cells grow as a separate form and do not induce homologous C3 deposition (17, 18). mAbs against human C3b (C-5G), C3bi (G-3E) (19), and MCP (M177) (20) were prepared as described. Anti-DAF mAb IA10 (21) was a gift from Dr. T. Kinoshita (Osaka University, Osaka, Japan). Rabbit polyclonal Ab against P39⁻ cells was prepared in our laboratory (Center for Adult Diseases, Osaka). A rabbit was immunized intraperitoneally with 10⁷ P39⁻ cells twice at 7-d intervals, followed by three intravenous injections with P39⁻ cells at 7-d intervals. After the last immunization, blood was drawn from an ear vein three times at 3-d intervals. IgG was purified by ammonium sulfate precipitation followed by protein A-Sepharose column chromatography, then coupled to BrCN-activated Sepharose 4B. FITC-conjugated goat F(ab)₂ of anti-mouse IgG was purchased from Cappel Laboratories (West Chester, PA), and HRP-labeled goat anti-mouse IgG was from Bio-Rad Laboratories (Richmond, CA). Mouse IgG and goat IgG were from Sigma Chemical Co. (St. Louis, MO). Recombinant human TNF-α (sp act 10⁵ U/ml) was a gift from Dainippon Seiyaku Co., Ltd. (Osaka, Japan).

Proteins, Buffers, and Human Serum. MCP was purified from K562 cells as described (22). DAF was purified from human erythrocyte membranes by butanol extraction followed by Q-Sepharose, hydroxylapatite, and anti-DAF mAb affinity column chromatography (13, 21). Human C3 was purified by the method of Nagasawa and Stroud (23), and methylamine-treated C3 (C3_{MA}) was prepared as described (24). SDS-PAGE marker proteins were purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan).

Gelatin veronal-buffered (GVB) saline containing 2 mM MgCl₂ and 10 mM EGTA (Mg²⁺-EGTA-GVB) or 10 mM EDTA (EDTA-GVB) was used in the assay of C3 deposition (18, 25). Normal human serum (NHS) was collected from 40 healthy donors and stored in aliquots at -70°C. A 1:20 volume of 40 mM Mg²⁺-200 mM EGTA (pH 7.4) or 200 mM EDTA (pH 7.4) was added to NHS to prepare Mg²⁺-EGTA-NHS or EDTA-NHS.

Preparation of a mAb Against a C3-activating Molecule on P39⁺ Cells. The complexes of C3 fragments and C3 accepting membrane molecules were partially purified as follows. P39⁺ cells (5 × 10⁹) were incubated in 25% Mg²⁺-EGTA-NHS for 30 min at 37°C. Cells bearing C3 were washed and solubilized with 1% NP-40/DPBS containing 10 mM EDTA, 25 mM iodoacetamide, and 2 mM PMSF for 30 min at room temperature and centrifuged at 10,000 g for 20 min. The supernatants were applied to a polyclonal anti-P39⁻ cell Ab-coupled Sepharose column equilibrated with PBS containing 0.05% NP-40 and 0.2 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (*p*-APMSF). The pass-through fraction was then applied to an anti-C3b mAb-coupled Sepharose column equilibrated with the same buffer. The column was sequentially washed with equilibration and high salt buffers (containing 0.6 M NaCl). The complexes with C3 fragments and membrane proteins were eluted with 0.17 M glycine-HCl buffer, pH 2.5, containing 0.05% NP-40 and 0.2 mM *p*-APMSF, dialyzed against 0.05 M ammonium bicarbonate/0.01% SDS, then lyophilized.

mAb was produced by the method of Köhler and Milstein (26). 8-wk-old female BALB/c mice were subcutaneously immunized with partially purified complexes of C3 fragments and membrane proteins in CFA three times, followed by two subcutaneous injections of the complexes in IFA in a 2-mo period. 10 d later, the mice were injected intraperitoneally with 10⁷ P39⁺ cells. For the final booster, 10⁷ P39⁺ cells were given intraperitoneally 1 wk after the cell injection. 3 d later, the spleen was extracted, and the cells were fused with mouse myeloma cell line NS-1 (26).

Hybridoma clones that secreted antibodies against P39⁺ cell membrane molecules but not P39⁻ cells or human C3 fragments were selected by subtractive screening by use of the protein A-rossette assay (27). The clones producing a mAb that reacted with P39⁺ but not P39⁻ cells were additionally confirmed by flow cytometry using the two sublines. The selected hybrids were cloned by limiting dilution. The mAb M161(γ₁, κ), was obtained and purified from mouse ascites by ammonium sulfate precipitation followed by DEAE-Sepharcel ion exchange chromatography (28).

Purification of M161 Ag. P39⁺ cells (5 × 10⁹) were solubilized with 1% NP-40, 25 mM iodoacetamide, 10 mM EDTA, and 2 mM PMSF in DPBS for 30 min at room temperature. Cell lysates were centrifuged at 10,000 g for 10 min, then the supernatants were diluted to 1:2.5 with ice cold PBS containing 0.5 mM *p*-APMSF and applied to mouse IgG-coupled Sepharose equilibrated with PBS/0.02% NP-40/0.2 mM *p*-APMSF. The pass-through fraction was dialyzed against 20 mM PB/20 mM NaCl/0.02% NP-40/0.2 mM *p*-APMSF, pH 7.5, overnight at 4°C, then applied to a Q-Sepharose column (2.5 × 15 cm) equilibrated with the same buffer. The column was washed with the starting buffer and eluted with a linear NaCl gradient (20–500 mM) in 500 ml of starting buffer. The M161 Ag was monitored by immunoblotting with the M161 Ab, and detected in the pass-through fraction. The pH of the unbound fraction was adjusted to 6.0 and dialyzed overnight against 20 mM PB/0.02% NP-40/0.2 mM *p*-APMSF, pH 6.0. Insoluble materials were removed by centrifugation at 10,000 g for 20 min, and the supernatant was applied to S-Sepharose (column 2.5 × 15 cm) equilibrated with the same buffer. After the column

was washed with the starting buffer, the sample was eluted with a linear salt gradient of the starting buffer and 500 mM NaCl in the same buffer (300 ml each). The eluates containing the M161 Ag were pooled, adjusted to pH 9.4, and dialyzed against 10 mM Tris-HCl/0.05% NP-40/0.2 mM *p*-APMSF, pH 9.4. A small amount of the precipitate was generated during dialysis, from which the insoluble material was separated by centrifugation at 10,000 *g* for 20 min and dissolved in PBS/0.05% NP-40/0.2 mM *p*-APMSF. The supernatants were applied to a chromatofocusing column (2 × 80 cm) equilibrated with 10 mM Tris-HCl/0.05% NP-40/0.1 mM *p*-APMSF, pH 9.4. The column was washed with 20 mM acetate buffer/0.05% NP-40/0.1 mM *p*-APMSF, pH 6.0, in 10% (vol/vol) Polybuffer 96 (Pharmacia, Uppsala, Sweden). The M161 Ag was eluted at a pH ranging from 9.3 to 9.4.

Deglycosylation of M161 Ag. The M161 Ag in 20 mM Tris-maleate buffer, pH 6.0, containing 10 mM D-galactono- γ -lactone, 1 mM calcium acetate, and 0.1% NP-40 was incubated with 50 mU of neuraminidase (Sigma Chemical Co.) for 1 h at 37°C, followed by incubation with 3 mU of O-glycanase (Genzyme Corp., Boston, MA) for 16 h at 37°C. N-glycanase digestion was performed by incubating M161 Ag in PBS/0.05% NP-40 with 24 mU of N-glycanase (Genzyme Corp.) for 16 h at 37°C (22).

SDS-PAGE and Western Blotting. SDS-PAGE was performed as described (29) under nonreducing and reducing conditions. After electrophoresis, the resolved proteins were transferred to nitrocellulose (30). The sheet was then blocked with 10% skim milk for 1 h at 37°C, then overnight at 4°C, and sequentially incubated

with the appropriate mAb, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and substrate for HRP (Konica Co., Tokyo, Japan).

Flow Cytometry. Cells (10⁶) suspended in 50 μ l of DPBS containing 0.5% BSA (BSA/DPBS) were mixed with 50 μ l of EDTA-plasma and 5 μ g of mouse IgG or mAb and incubated for 30 min at 4°C. After washing with BSA/DPBS, the cells were suspended in 90 μ l of BSA/DPBS and incubated with FITC-conjugated goat F(ab)₂ of anti-mouse IgG at 4°C. After 30 min, the cells were washed twice with DPBS and fixed with paraformaldehyde. The samples were analyzed on a cytofluorograph (EPICS Elite, Coulter Corp., Hialeah, FL).

C3 deposition on P39 cells was examined as described (19). Briefly, 10⁶ cells were incubated with 25% Mg²⁺-EGTA-NHS or EDTA-NHS for 30 min at 37°C. After washing, C3 fragments bound to the cells were detected with anti-human C3b mAb and FITC-labeled second Ab.

Trypsin Digestion. P39⁺ cells (5 × 10⁶) suspended in 2 ml of DPBS were incubated with various doses of trypsin (Sigma Chemical Co.) or buffer alone for 2 min at 37°C. The reaction was stopped by adding 10 ml of 10% FCS-RPMI. After centrifugation at 1,000 *g* for 6 min, the cells were washed once with 10% FCS-RPMI and then 0.5% BSA-DPBS. The levels of C3 deposition, M161 Ag, MCP, and DAF were determined by flow cytometry.

Functional Analysis of M161 Ag. Purified MCP, DAF, and M161 Ag were resolved by SDS-PAGE (10% gel) under nonreducing conditions and electroblotted onto nitrocellulose. The sheet was cut

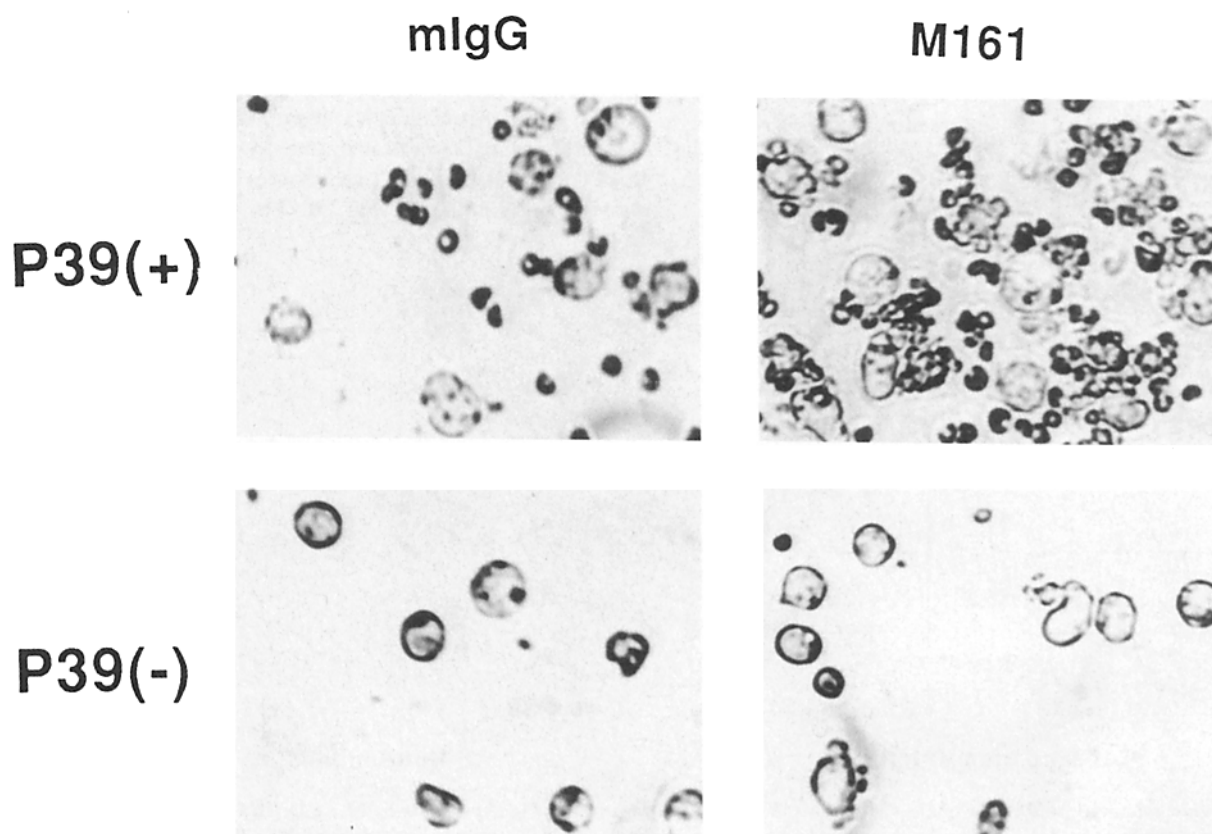


Figure 1. Protein A-rosette assay for screening mAb recognizing P39⁺ but not P39⁻ cells. The supernatants of hybridoma clones (see Materials and Methods) capable of recognizing P39⁺ but not P39⁻ cells were first selected by protein A-rosette assay, and the hybridoma cells were subjected to limiting dilution. One mAb, named M161 Ab, was established by this method. The rosetting profile of this mAb with P39⁺ cells is shown (upper right). The reactivity of this mAb with P39⁻ cells (lower right) and that of control nonimmune mouse IgG with P39^{+/-} cells (left) are shown.

into strips, each of which was blocked with 10% skim milk for 1 h at 37°C, then overnight at 4°C. The strips were incubated with 25% EDTA-NHS, Mg²⁺-EGTA-NHS, or C3_{MA} (50 µg/ml) for 60 min at 37°C. After three washes with PBS/0.05% Tween 20, the strips were soaked with anti-human C3b mAb, anti-human C3bi mAb, or control mAb, followed by HRP-labeled goat anti-mouse IgG to detect the deposited C3 fragments. The purity of MCP, DAF, and M161 Ag and reactivity with each mAb were confirmed by SDS-PAGE followed by silver staining or Western blotting.

When hydroxylamine was used, the M161 Ag electroblotted onto nitrocellulose was incubated in 0.1 M NaHCO₃, pH 10, with 1 M hydroxylamine (31) for 2 h at 37°C after the incubation with 25% Mg²⁺-EGTA-NHS. Since the ester bond was hydrolyzed in the basic buffer, pH 10 even without hydroxylamine, as described previously (32), DPBS (pH 7.4) was used as a control buffer. Binding of C3 fragments to the M161Ag was detected with anti-human C3b mAb and HRP-labeled second antibody.

Results

Establishment and Properties of mAb M161. By repetitive subtractive screening using the two P39 sublines, we obtained one mAb, M161 (γ₁, κ), which recognized the cell surface molecule expressed on P39⁺ but not P39⁻ cells (Fig. 1). The flow cytometric profile of M161 Ab-stained P39⁺ cells was not unimodal, suggesting cell-to-cell variation of the expression degree of the M161 Ag (Fig. 2). The M161 Ag was expressed in P39⁺ but not in P39⁻ cells, while other surface markers, including membrane C regulatory proteins, MCP, and DAF, were similarly expressed on both cell lines, consistent with previous results (18). Intercellular adhesion molecule 1 was expressed, though marginally, in P39⁺ but not in P39⁻ cells, which was the only known phenotypic differ-

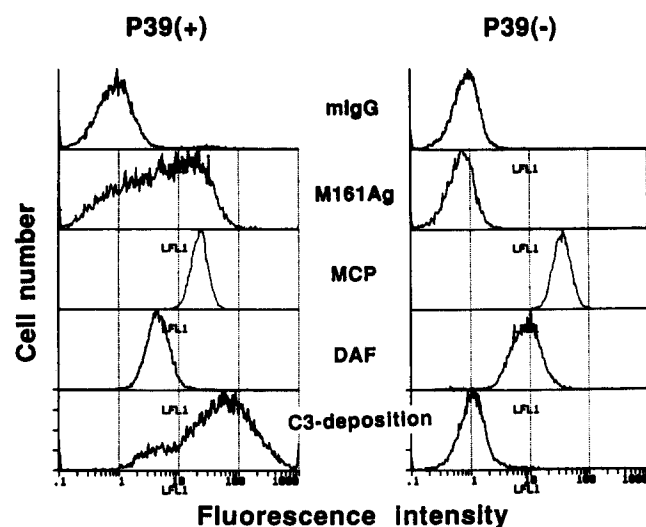


Figure 2. Flow cytometry of M161 Ag on the P39 sublines. The M161 Ab established as described in the legend to Fig. 1 was used to detect the corresponding Ag by flow cytometry. The level of M161 Ag along with those of DAF and MCP expressed on P39⁺ and P39⁻ cells was detected with each mAb and FITC-labeled goat anti-mouse IgG. C3 deposition was simultaneously assessed by use of the P39 cells treated with Mg²⁺-EGTA-NHS, and anti-human C3b mAb and FITC-labeled second Ab.

Table 1. Reactivity of mAb M161 with Hematopoietic Cell Lines*

Cell line type	M161 Ag positive	M161 Ag negative
Myeloid	P39 ⁺	P39 ⁻ , HL60, U937, THP1, KG1, KY821, KU812
B lymphoid		Daudi, P32, Ramos, Raji, CCRF-SB
T lymphoid		MT1, TALL1, CCRF-CEM, HSB2, HLCL1, Molt4
Erythroblastoid		K562, HEL

* Surface expression of the M161 Ag on cell lines was analyzed by flow cytometry as described in Materials and Methods.

ence between the two sublines (see reference 18). During the culture interval, the level of the M161 Ag was varied in accordance with the C3 activating ability in P39⁺ cells, while expression of other cell surface molecules, including DAF, MCP, and LFA-1 did not change (data not shown). As shown in Table 1, all cell lines except for the P39⁺ subline tested to date were M161 Ag negative; although not shown in Table 1, some premature bone marrow cells, particularly promyelocytes and metamyelocytes, were M161 Ag positive.

Immunoblotting indicated that M161 Ab recognized a 43-kD molecule in P39⁺ cell lysates under reducing and nonreducing conditions (Fig. 3). The lysates of P39⁻ cells

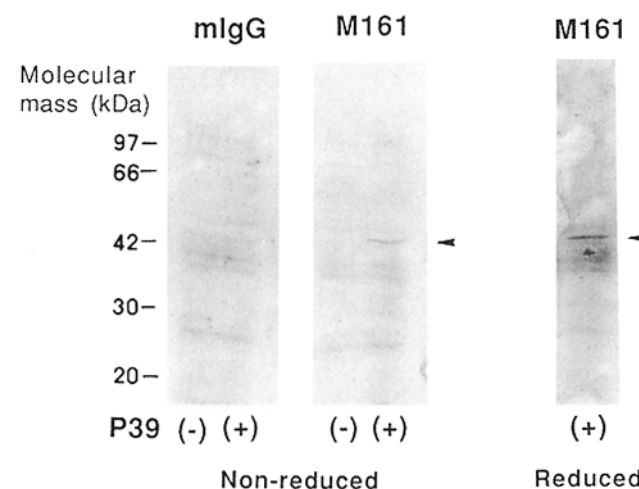


Figure 3. Analysis of the M161 Ag by SDS-PAGE/immunoblotting. P39⁺ and P39⁻ cells were solubilized with 1% NP-40. Samples were resolved by SDS-PAGE (10% acrylamide) under nonreducing (left and center) or reducing (right) conditions and electroblotted onto nitrocellulose. Immunoblotting was performed by use of nonimmune mouse IgG (left) or the M161 Ab (center and right) for the first Ab and HRP-labeled goat anti-mouse IgG. The arrow indicates the M161 Ag.

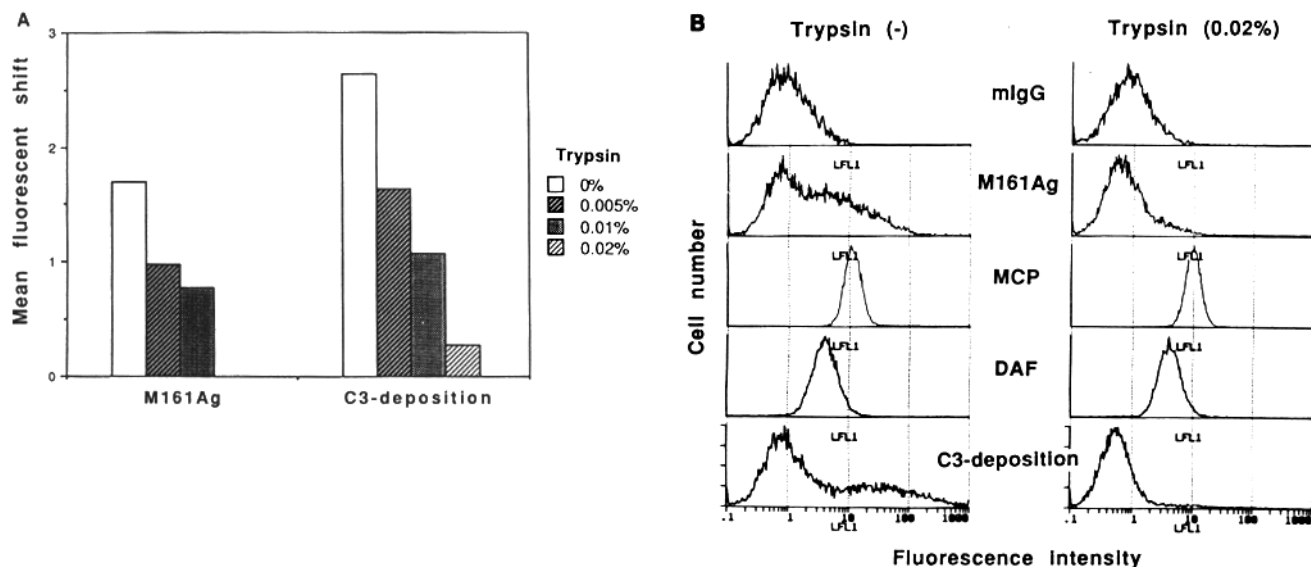


Figure 4. The decrease of M161Ag and C3 deposition in P39⁺ cells induced by trypsin. (A) Decrease of C3 deposition in P39⁺ cells and the disappearance of the cell surface M161 Ag paralleled the function of trypsin. P39⁺ cells were incubated with 0 (□), 0.005 (■), 0.01 (▣), and 0.02% (▤) trypsin for 2 min at 37°C. After the reaction was stopped by adding 10% FCS-RPMI, the cells were washed, and the levels of M161 Ag and C3 deposition were assessed by flow cytometry. (B) Effect of trypsin (0.02%) on MCP and DAF expressed on P39⁺ cells. P39⁺ cells were incubated with 0.02% trypsin or control buffer [Trypsin (-)] then analyzed by flow cytometry using anti-MCP and anti-DAF mAbs. Levels of M161 Ag and C3 deposition before and after trypsin exposure are also shown.

and other myeloid cell lines did not contain the 43-kD protein (data not shown).

The relationship between the expression of the M161 Ag and C3 activating ability of P39⁺ cells was further studied. P39⁺ cells were treated with different doses of trypsin, and the levels of the M161 Ag and homologous C3 deposition were assessed by flow cytometry. The C3-activating ability of P39⁺ cells diminished in parallel with the dose of trypsin and disappearance of the cell surface M161 Ag (Fig. 4 A).

The levels of MCP and DAF were not affected by trypsin (Fig. 4 B). In another experiment, TNF- α increased the level of M161 Ag, and the degree of C3 deposition was again increased accordingly (Fig. 5). These results suggested that the M161 Ag is closely associated with homologous C3 activation on P39⁺ cells.

Purification of the M161 Ag from P39⁺ Cells. Because of the low affinity of the M161 Ab to the antigen in the P39⁺ solubilized preparation, immunoaffinity was not a suitable

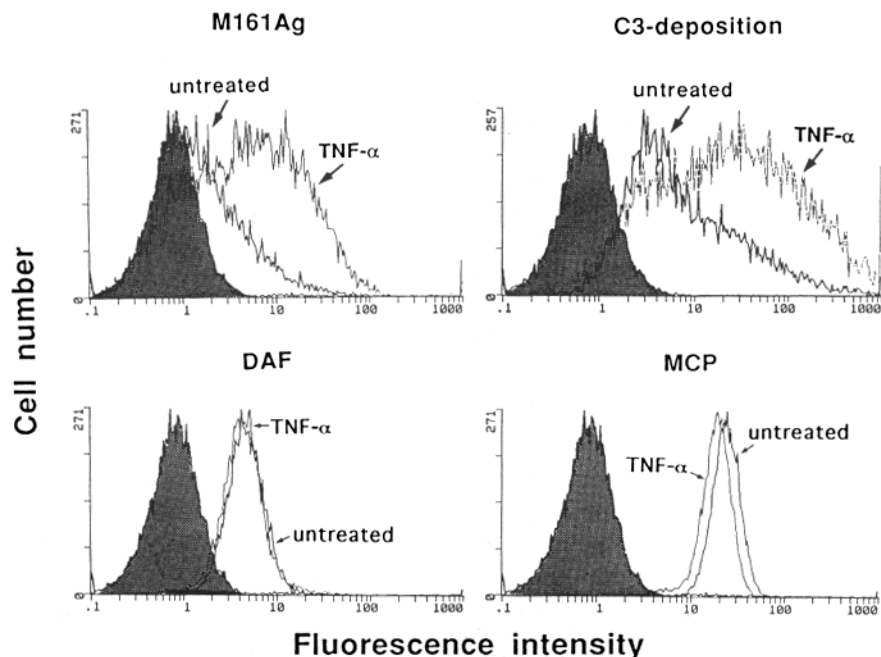
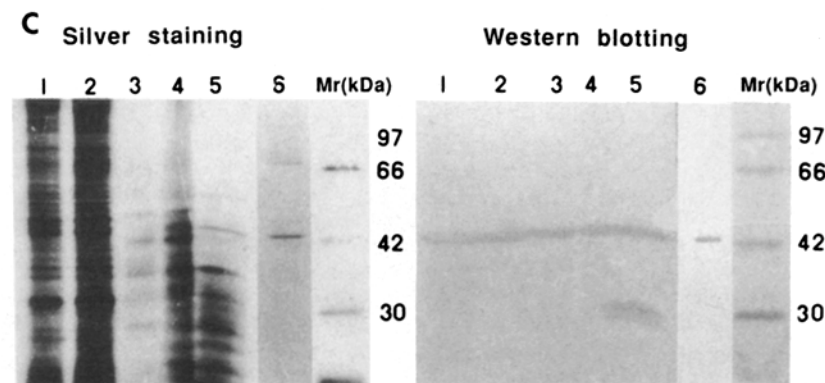
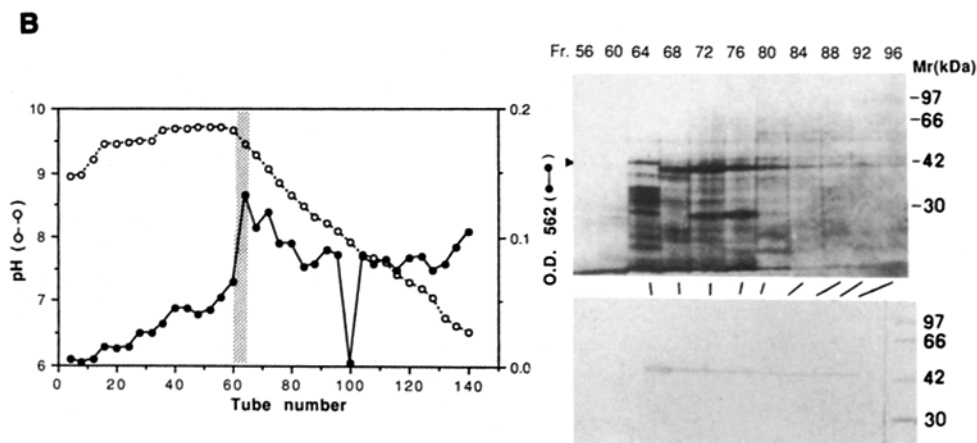
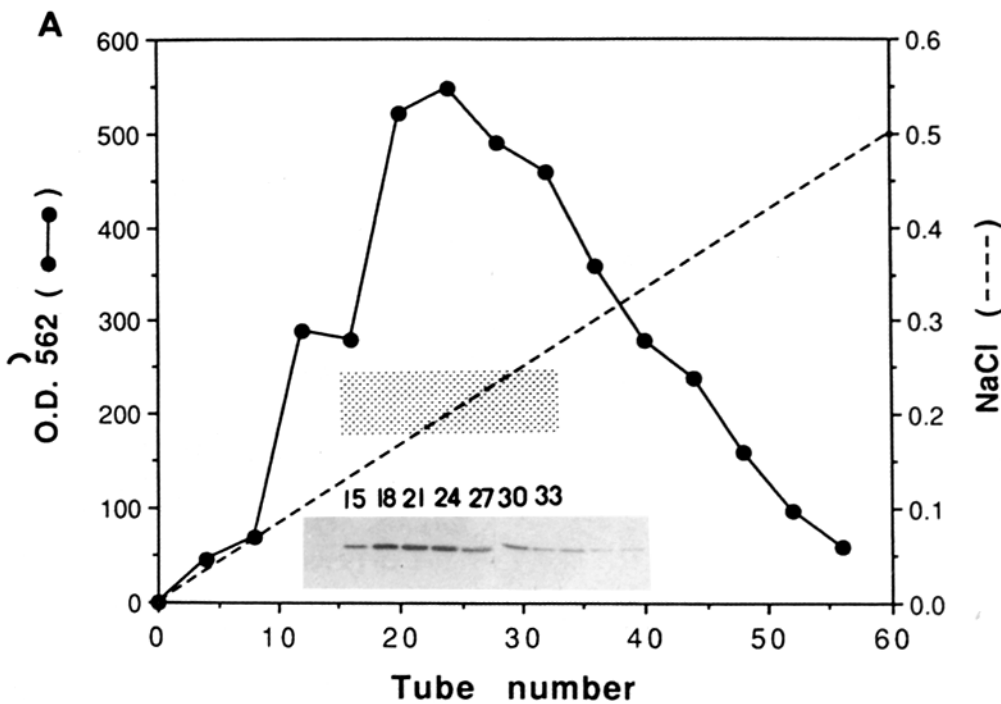


Figure 5. Upregulation of M161 Ag and C3 deposition on P39⁺ cells by TNF- α . P39⁺ cells cultured in medium alone (*untreated*) or in 50 U/ml of TNF- α were harvested after 48 h, and then the levels of M161 Ag, DAF, and MCP were analyzed by flow cytometry using, respectively, mAbs and FITC-labeled second antibody. The level of C3 deposition was also assessed by flow cytometry using anti-human C3b mAb after the cells were treated with 25% Mg²⁺-EGTA-NHS or 25% EDTA-NHS. Shaded histograms are control P39⁺ cells treated with nonimmune mouse IgG and second antibody or those treated with EDTA-NHS, and then with anti-C3b mAb (*upper right*). TNF- α induced marked upregulation of the levels of M161 Ag and C3 deposition but only marginal change in the levels of MCP and DAF. Three experiments were performed and a representative one is shown.



1. P39(+) cell lysate
2. mIgG-sepharose pass through
3. Q-sepharose pass through
4. S-sepharose eluate
5. Chromatofocusing eluate
6. Isoelectric point precipitate

Figure 6. Purification of the M161 Ag. (A) Elution profile of the M161 Ag from S-Sepharose. The pass-through fraction from Q-Sepharose (C) was applied to S-Sepharose and eluted with a linear NaCl gradient. The protein concentration was determined in each fraction by use of the Pierce protein assay kit and is shown as OD_{562 nm}. The M161 Ag was monitored by immunoblotting, and the result is shown in the inset (fraction numbers are indicated). (B) Elution profile of the M161 Ag from a chromatofocusing gel. The eluate from S-Sepharose was dialyzed against the buffer for chromatofocusing, and then applied to the column. The M161 Ag monitored by immunoblotting was apparent at pH 9.3-9.4, and then it tailed to the following fractions. Protein determination (left) and immunoblotting (lower right) were performed with each fraction as in A. The eluates were also silver stained (upper right). The arrow indicates the band corresponding to the M161 Ag. (C) Silver staining and immunoblotting profiles of each purification step. Column fractions in each step were resolved by SDS-PAGE (10% gel) under nonreducing conditions followed by silver staining (left). In the right panel, the same fractions were analyzed by SDS-PAGE/immunoblotting by use of the M161 Ab and HRP-labeled second antibody. In lane 5, a doublet of 30-kD bands is present besides the main 43-kD band. This is a degradation product, since this 30-kD moiety was increased during storage at 4°C.

means of purification. We therefore used ion exchange chromatography, chromatofocusing, and isoelectric point (pI) precipitation to purify M161 Ag. The solubilized preparation was first loaded onto mouse IgG-coupled Sepharose and then onto a Q-Sepharose column. M161 Ag was monitored by silver staining and Western blotting using the M161 Ab. M161 Ag did not bind Q-Sepharose under our conditions. The pass-through fraction from Q-Sepharose (Fig. 6 C, lane 3) was applied to an S-Sepharose column. M161 Ag bound S-Sepharose and was eluted with NaCl at concentrations ranging from 120 to 300 mM (Fig. 6 A). A portion of the M161 Ag-positive fractions (Fig. 6 C, lane 4) were chromatofocused to determine the pI of the Ag. M161 Ag was eluted with Poly buffer 96 at pH 9.3 to 9.4, although tailing was evident. As shown in Fig. 6 B, the eluates from the chromatofocusing column still contained a considerable amount of low molecular weight proteins (see also Fig. 6 C, lane 5). Since the pI of the M161 Ag was determined at this step, we tried to recover the protein by pI precipitation. The pooled S-Sepharose fractions were dialyzed against pH 9.4 buffer overnight at 4°C. The precipitate generated during dialysis was separated by centrifugation, and SDS-PAGE showed that it largely consisted of M161 Ag (Fig. 6 C, lane 6). We used this material to further study M161 Ag. The two bands around 30-kD in lane 5 (right) appeared to be degradation products of M161 Ag, since they were increased during 4°C storage of the crude material.

Deglycosylation revealed that the M161 Ag did not possess N- or O-linked carbohydrates (data not shown).

Functional Analysis of M161 Ag. Purified M161 Ag was resolved by SDS-PAGE under nonreducing conditions and electroblotted onto nitrocellulose. Each strip of nitrocellulose was incubated with EDTA-NHS or Mg²⁺-EGTA-NHS, then bound C3 fragments were detected with anti-human C3b or anti-human C3bi mAb and HRP-labeled second antibody. C3 fragments efficiently bound to the M161 Ag during incubation with Mg²⁺-EGTA-NHS but not to purified MCP or DAF, which were control C3b-binding membrane

proteins similarly blotted and incubated. The anti-C3b mAb predominantly bound to the 43-kD portion compared with the anti-C3bi mAb, suggesting that C3b is a major species of the bound C3 fragments. No C3 fragments bound M161 Ag in the presence of EDTA-NHS (Fig. 7). Thus, human C3b binds selectively to the purified M161 Ag via activation of the alternative pathway in a manner resistant to factors H and I in NHS.

We next examined which type of linkage sustained the M161 Ag-C3b complex. The repetitive washing of the strip with high salt buffer did not reduce the C3b deposition. C3 possesses a thioester bond in its α chain, which mediates the covalent linkage of C3b with target molecules (2). To clarify this issue, complexes of M161 Ag and C3 fragments trapped onto nitrocellulose paper were exposed to hydroxylamine. As shown in Fig. 8, the amount of the C3 fragments bound to the M161 Ag was decreased ~40% by hydroxylamine. Additionally, methylamine-treated C3 (C3b analogue) or native C3 (data not shown) did not bind the M161 Ag (Fig. 8 A). These results indicate that the C3 fragments are linked to the M161 Ag at least partly via the specific ester bond formed secondarily to the activation of the alternative complement pathway. The epitope recognized by the M161 Ab may not be in close proximity to the acceptor site for C3b on the M161 Ag, since the M161 Ab neither blocked the deposition of C3b onto the M161 Ag nor lost the reactivity with the M161 Ag after the deposition of C3 fragments (Fig. 8).

Discussion

These studies were initiated because unidentified molecules on the human P39 subline P39⁺ activated the human alternative C pathway, and allowed the deposition of homologous C3 on its membrane (17, 18). Human cells are usually protected from homologous C attack, including the key step, C3 deposition, by expressing C regulatory proteins. Membrane DAF and MCP and plasma factors H and I are representative of factors responsible for self-protection against the

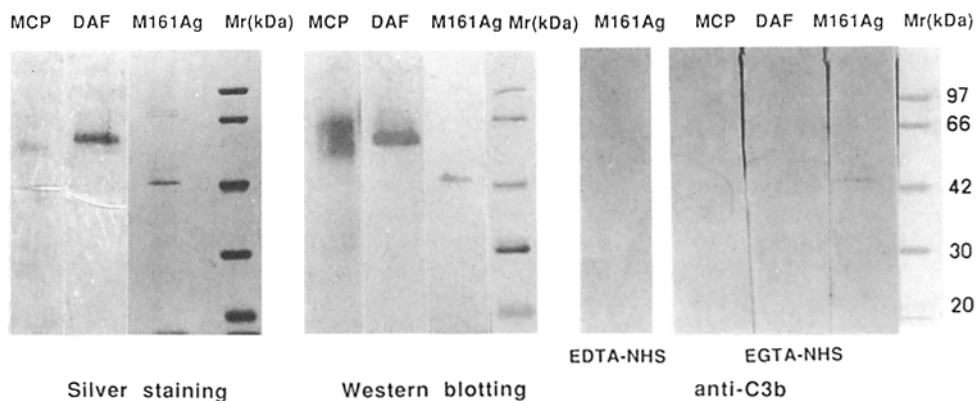


Figure 7. The ability of M161 Ag to activate C3 and allow the deposition of C3 fragments. Four sets of the purified K562 MCP, human erythrocyte DAF, and M161 Ag (Fig. 6, lane 6) were simultaneously resolved by SDS-PAGE (10% acrylamide) under nonreducing conditions. One of them was stained by use of the Bio-Rad silver staining kit (left), and three of them were electroblotted onto nitrocellulose. One of the three transblotted sets was incubated with 25% EDTA-NHS and one with Mg²⁺-EGTA-NHS for 60 min at 37°C. After washing, the nitrocellulose was in-

cubated with anti-human C3b mAb and HRP-labeled second antibody to detect the deposited C3 fragments (right). Although not shown in the figure, EDTA-NHS did not allow the deposition of C3 on DAF or MCP. The other set was analyzed by Western blotting with mAb M177, IA10, and M161 Ab, respectively (center).

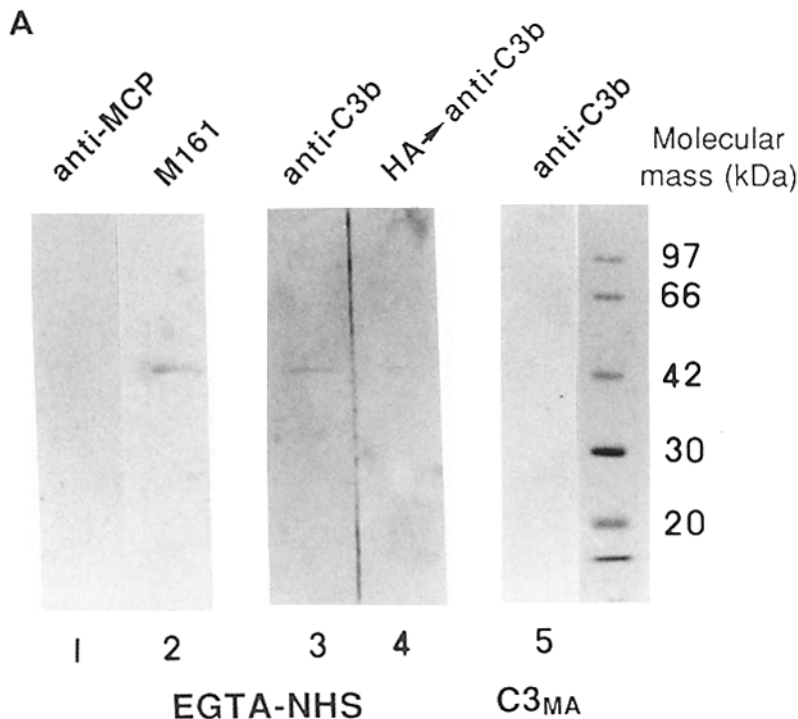


Figure 8. (A) The detachment of C3 fragments from the M161 Ag by hydroxylamine. The M161 Ag electroblotted onto nitrocellulose was incubated with 25% Mg^{2+} -EGTA-NHS (lanes 1–4) or $C3_{MA}$ (lane 5) for 60 min at 37°C. After washing, one set of nitrocellulose strips was incubated in DPBS (lane 3) or 1 M hydroxylamine (HA), pH 10, (lane 4) for 2 h at 37°C. The binding of C3 fragments to M161 Ag was then detected with anti-C3b mAb (lanes 3–5). The other set of strips was reacted with anti-MCP mAb (lane 1) or M161 Ab (lane 2). (B) Determination of the diminution of the C3b deposited on M161 Ag by hydroxylamine treatment. C3 fragments were fixed on the M161 Ag blotted onto nitrocellulose as in A. Densitometric analysis was performed for the assessment of the C3 fragments deposited onto the M161 Ag. The levels of C3 fragment deposition in control samples were assumed to be 100%. The bound C3 was decreased by ~40% by hydroxylamine. Two additional experiments are shown. Although not shown in the figure, ~20–30% decrease was observed with the pH 10 buffer alone. (See next page.)

classical and alternative pathways of homologous C; therefore, C3 deposition is strictly prohibited, although human cells are exposed to an abundant plasma C source (2, 32). Thus, the molecules that activate homologous C on P39 membrane permit the deposition of C3, thus circumventing the regulatory action of DAF and MCP.

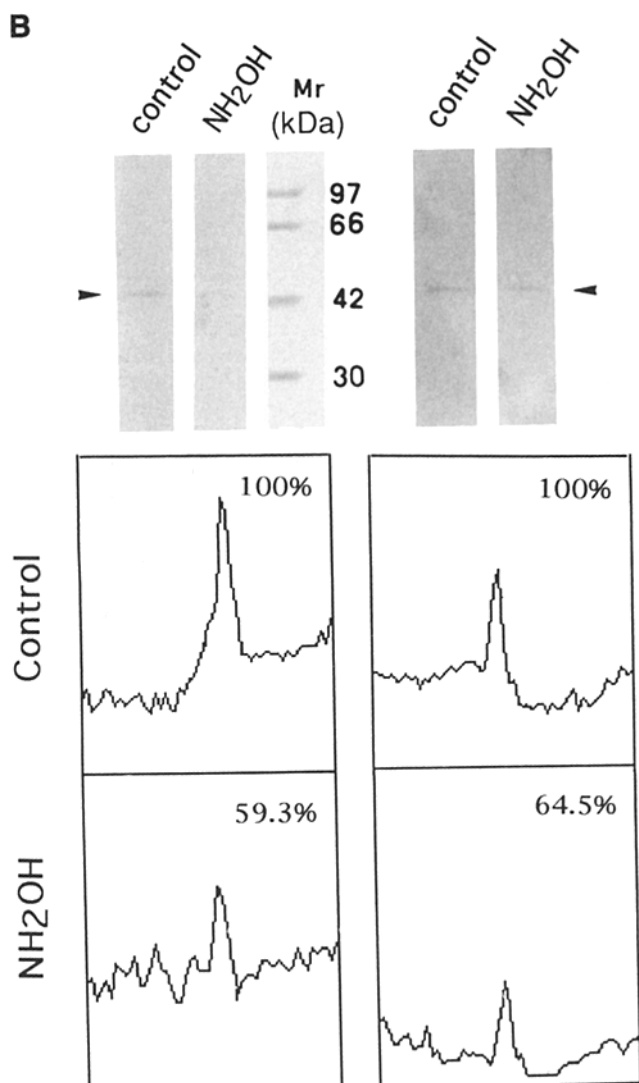
We found that 90-, 60-, and 40-kD proteins of P39⁺ cells serve as C3-accepting molecules (18). Judging from the relative molecular mass, the M161 Ag is the reported 40-kD protein. Although the possibility is not excluded that the other two are involved in homologous alternative pathway activation, we consider that the M161 Ag is a C3-activating protein. This idea is supported by several lines of evidence: (a) no tested cell lines, including the P39⁻ sublines, which fail to induce C3 deposition, express the M161 Ag (Table 1); (b) the M161 Ag transblotted onto nitrocellulose allows the deposition of the C3 fragment, C3b, on itself via ester linkage (Figs. 7 and 8); (c) the disappearance of M161 Ag secondary to trypsin digestion is in accordance with a decrease of C3 deposition in P39⁺ cells (Fig. 4); (d) the expression level of the M161 Ag and the degree of C3 deposition are regulated in parallel by TNF- α (Fig. 5); and (e) M161 Ag appeared not to be a protease that activates C3 directly, since exposing M161 Ag with various protease inhibitors did not suppress C3 deposition.

During the investigation of the M161 Ag, the possibility was raised that this molecule is an “activator” of foreign origin. We therefore examined the following. First, chromosomal analysis and a viral contamination check were performed with our P39 cell lines in Japanese Cancer Research Resources Bank, and we confirmed that the cells were unequivocally of human origin and without viral infection. Second, the cells were not

targeted by homologous Ab, suggesting that this is not Ab-dependent activation of the alternative pathway (18). Third, homologous C activation and subsequent C3 deposition occurred on this molecule in the absence of Ca^{2+} (Fig. 7), thus negating the involvement of the lectin pathway (33, 34). These findings, together with the fact that the M161 Ag is present in bone marrow cells in premature stages, reinforce the notion that this “activator” is a human gene product.

A similar activator-like functional feature has been found in human CR2 in some lymphoblastoid cell lines (7–9). Indeed, a covalent complex of C3b–CR2 has been identified in the Burkitt’s lymphoma cell line, Raji (8). However, the M161 Ag we discovered is distinct from CR2 or its fragment, since (a) the molecular mass of the M161 Ag was 43 kD while that of CR2 is ~140 kD (35); (b) P39⁺ cells did not express CR2 (18); (c) M161 Ab did not recognize CR2 (data not shown); and (d) NH₂-terminal analysis revealed that the M161 Ag is an unregistered protein (data not shown). Hence, the M161 Ag is a novel human membrane protein with alternative C pathway-activating ability. Studies on the structure, function, and physiological role of this protein are in progress.

M161 Ab did not block C3b deposition or C3-activating ability of the M161 Ag. Several groups have suggested that metastable C3b bound covalently to a specific portion of membrane-bound biological molecules such as IgG (36), C4b (37, 38), and C3b (39). It is important for the protein complex to maintain a certain tertiary structure for the expression of the C3/C5 convertase activity (37–39). Of note, these C3b-accepting molecules all belong to the Ig superfamily, short consensus repeat (SCR), or complement C3b/C4b. Although at present we only have information about the NH₂-terminal sequence of the M161 Ag, it might also be a member



of these families and possess a specific hydroxyl group as a C3b-accepting site. If so, it is not surprising that the M161 Ab does not block the deposition of homologous C3b, because the epitope of this mAb may not be present at close proximity to the C3b-accepting portion.

Granulocytes infiltrate inflammatory areas via binding to C3b/C3bi deposited on the damaged endothelial cells (40). C receptors CR1 and CR3 on granulocytes reportedly participate in this cell infiltration (40, 41). The importance of C and its receptors for malignant cell invasion has also been shown in previous reports (42). The molecules responsible for C3 deposition under these conditions remain unidentified. Another report has suggested that C3 deposition physiologically occurs in damaged cells for facilitating clearance by C receptor-positive leukocytes (43–46). In fact, P39⁺ sublines were established through culture in conditions under which most cells died (18). These findings may confer physiological importance upon C3 deposition. It contributes to cell invasion; otherwise, it facilitates phagocytosis for the clearance of cell debris. We present the notion that M161 Ag is one of the proteins related to these functions.

Regardless, C3 deposition is a likely event in cells of certain conditions and is strictly regulated in normal cells. C regulatory proteins are reportedly important for such regulation to maintain homeostasis of C activation and to be a safeguard against cell surface C activation (10–14, 47, 48). In contrast, here we surmise that not only C inhibitors but also activators have a relevant part in homologous C activation and regulation on human cell surfaces, the balance of which solidifies the maintenance of C homeostasis.

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Note added in proof: The concept that human cells/cell lines can be targets for homologous complement has also been proposed by recent papers (49, 50) after the submission of this work.

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